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ARTICLE

Genetic characterization of Egyptian buffalo CSN3 gene

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Abstract Genetic polymorphism k-casein (CSN3) gene was investigated in lactating Egyptian buffalo using nucleotide sequencing. Primer pairs amplified a 453 nucleotide fragment of CSN3 exon IV with an open reading frame of 421 nucleotides encoding 139 amino acids of the mature peptide and 32 nucleotides 3′UTR. Two SNPs (nt-315 C/T and nt-319 C/T) occurred in amplified fragment. These SNPs were reflected at codon 105 (ACC/ATC) and codon 106 (ACC/ACT) which correspond to codon 135 and 136 of the CSN3 mature peptide, respectively. Variation at codon 135 caused a change from ACC (Threonine) versus ATC (Isoleucine) whereas variation at codon 136(Thr/Thr) is a silent mutation. The results show, contrary to previous reports, that Egyptian buffalo has both alleles A (135^{Thr}ACC/136^{Thr}ACC) and B (135^{Ile}ATC/136^{Thr}ACT) with allelic frequencies of 0.57 and 0.43, respectively. The Egyptian buffalo genotype frequencies were 0.294 (AA), 0.647 (AB) and 0.058 (BB). The polymorphic site at codon 135 (A[C/T]C) and 136 (AC[C/T]) has C bases in allele A and T bases in allele B, resulting in two haplotypes; $135^{Thr(ACC)}$/$136^{Thr(ACC)}$ and $135^{Ile(ATC)}$/$136^{Thr(ACT)}$. The frequency of the former haplotype was 0.57. In this study we investigated the reason why buffalo samples, analyzed by RFLP technique, using HindIII and HinfI used in cattle, were mistakenly identified as BB monomorphic. We suggest the use of restriction enzymes AcuI or Eco57MI to be used in buffalo CSN3 RFLP analysis. Digestion of buffalo CSN3-exon VI fragment (453 bp) with either enzyme will generate two fragments of 339 bp and 114 bp in allele B.

1. Introduction

The domestic water buffalos are main source of meat and milk. Their worldwide population is around 185 million [14]. They can bear different environmental and nutritious changes and are more resistant to disease than cow. They have great potential for genetic improvement in both milk and meat production. There are two types of buffaloes: the river buffalo ($2n = 50$) is found in the Indian Subcontinent, Middle East and Eastern Europe whereas the swamp buffalo ($2n = 48$) is distributed in China, Bangladesh, the Southeast Asian countries and North-Eastern states of India [7]. Interest in investigating the genetic potential of buffalo has led to the development of a physical genetic map [13,10] followed by the cytogenetic map [11] and radiation hybrid map [2].

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The Egyptian water buffalo population is around 3.8 million heads [15]. They are river buffalo and belong to Mediterranean buffalo which include those of Italy, Greece, Bulgaria, Syria and Turkey [26,18]. River buffalo were introduced to north Egypt after the ninth century [34] and have ever since become an important domestic animal. Over 95% of the Egyptian buffalo are kept in small holding farms with two to three buffalo in a herd [16,17].

Milk trait is controlled by several genes and among them are the casein genes. There are 4 casein genes which are tightly linked CSN1S1, CSN1S2, CSN2, and CSN3. They code for α(S1) and α(S2), β and K casein respectively. Kappa casein constitutes about 80% of total milk protein. The total gene length is around 13 kb. It constitutes of 5 exons coding for 190 amino acids, the first 21 a.a. residues constitute the putative signal peptide, whereas the mature peptide consists of 169 a.a. residues [9] from which 160 a.a. residues are in exon IV. Most of the studies investigating polymorphism in CSN3 involved exon IV. Mutations in exon IV are responsible for differences in gene expression [12].

CSN3 polymorphism in cattle has been extensively investigated. Thirteen protein variants and 1 synonymous variant have been reported in cattle CSN3 gene [19], however the most frequent ones are A and B alleles [29]. These alleles differ in two amino acid substitutions at codons 136 and 148 of the mature protein. Allele A has $136^{Thr(ACC)}/148^{Asp(GAT)}$, whereas allele B has $136^{Ile(ATC)}/148^{Ala(GCT)}$ [23].

In buffalo, CSN3 polymorphism has been investigated during the last decade using nucleotide sequence analysis. Two nucleotide variants at codons $135^{Thr(ACC)/Ile(ATC)}$ and $136^{Thr(ACC/ACT)}$ (silent mutation), have been reported in Italian [6], Bulgarian [5], and water buffalo genomic library [22]. On the other hand, several studies investigated polymorphism in buffalo using the PCR–RFLP method. Egyptian buffalo were reported to be BB monomorphic [28,8,21]. BB monomorphic buffaloes were also reported in Pakistan [31], Pandharpuri breed of India [33], South Kanara and Surti breed of India [20] and in Murrah breed of Brazil and their crossings [27]. All investigations, reporting BB monomorphic buffalo, were conducted by PCR–RFLP analyses using restriction enzymes such as, HindIII and HinfI used for investigating polymorphism in cattle CSN3.

The aim of this work was to genetically characterize the CSN3 gene in the Egyptian buffalo, investigate the reason behind BB monomorphism reported in buffalo using RFLP analysis and suggest a restriction enzyme suitable for buffalo RFLP analysis.

2. Material and methods

2.1. Blood samples and DNA extraction

Blood samples were taken from 17 lactating Egyptian buffalo. Genomic DNA was extracted from whole blood of buffalo by salting out method according to [24].

2.2. PCR amplification and nucleotide sequencing

The primers used for amplification of the CSN3 453 bp fragment were reported by [4] and have been used by [3,31] in cattle and in buffalo, respectively. They have the following nucleotide sequences F: 5′-3′ TGTGCTGAGTAGGTATCCTAGTTATGG; R: 5′-3′GCGTTGTCTTCTTTGATGTCTCCT. The expected 453 bp fragment of CSN3 covers most of the exon IV coding region.

Each amplification reaction (100 µl) contained 5 µl of buffalo DNA 50 ng/µl, 0.2 mM dNTPs, 10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin (W/V), 1.25 units Dream Taq polymerase (Thermo Scientific) and 1 µM primers. The reaction mixture was run in a Q-Cycler, Live Science. The following cycling conditions were used: 3 min. at 94 °C; 35 cycles for 1 min at 94 °C; 45 s at 60 °C; 80 s at 72 °C and a final extension for 10 min at 72 °C. PCR products were purified using GeneJET™ PCR Purification Kit (Fermentas #K0701) and sequenced by Bioneer, ABI 3730XL DNA analyser.

3. Results

CSN3 was investigated in 17 Egyptian lactating buffalo. The primer pair amplified a 453 bp fragment of exon IV. It covers 421 nts in coding region, and 32 nts were 3′UTR. The buffalo nucleotide sequences of the 17 samples were found to be identical except in 2 single nucleotide polymorphisms (SNP) at positions nt-315(C/T) and nt-319 (C/T) (Fig. 1). Variant bases at nucleotides 315 and 319 were similar. Five buffalo had C bases, one buffalo had T bases whereas eleven buffalo were heterozygous and they had C/T. The presence of C, T or C/T at both nt-315 and 319 has been verified in 33 additional buffalo (data not shown).

The amplified fragments (453 bp) showed an open reading frame of 420 nucleotides (frame 2) encoding for 139 amino acids (from 31 to 169 of the mature peptide) and a stop codon. The two identified SNPs at nt-315 and nt-319 in this study were present in codons 105 (A*C*C/A*T*C) and 106 (AC*C*/AC*T*) which correspond to codons 135 and 136 of the mature peptide and will be used hereafter. Codons 135 and 136 were translated into Threonine (Thr)/Isoleucine (Ile) and Threonine/Threonine, respectively, the latter is a silent mutation

```
1    Tgtgctgagtaggtatcctagttatggactcaattactaccaacag
       V  L  S  R  Y  P  S  Y  G  L  N  Y  Y  Q  Q  45
47   aaaccagttgcactaattaataatcaatttctgccataccatat
       K  P  V  A  L  I  N  N  Q  F  L  P  Y  P  Y   60
92   tatgcaaagccagctgcagttaggtcacctgcccaaattcttcaa
       Y  A  K  P  A  A  V  R  S  P  A  Q  I  L  Q   75
137  tggcaagttttgccaaatactgtgcctgccaagtcctgccaagcc
       W  Q  V  L  P  N  T  V  P  A  K  S  C  Q  A   90
182  cagccaactaccatgacacgtcacccacacccacatttatcattt
       Q  P  T  T  M  T  R  H  P  H  P  H  L  S  F  105
227  atggccattccaccaaagaaaaatcaggataaaacagaaatccct
       M  A  I  P  P  K  K  N  Q  D  K  T  E  I  P  120
272  accatcaataccattgttagtgttgagcctacaagtacaccta@c
       T  I  N  T  I  V  S  V  E  P  T  S  T  P  @  135
317  ac@gaagcaatagagaacactgtagctactctagaagcttcctca
       T  E  A  I  E  N  T  V  A  T  L  E  A  S  S  150
362  gaagttattgagagtgtacctgagaccaacacagcccaagttact
       E  V  I  E  S  V  P  E  T  N  T  A  Q  V  T  165
407  tcaaccgtcgtctaaaaactctaaggagacatcaaagaagacaac
       S  T  V  V  *                                169
452  gc
```

Figure 1 Nucleotide and amino acid sequences (frame 2) of Egyptian buffalo CSN3 amplified fragment. @ and @in nucleotide sequence are C bases in 5 animals, T bases in one animal and C/T heterozygous in eleven animals. @in amino acid sequence is Threonine in 5 animals, Isoleucine in one animal and Threonine/Isoleucine in 11 animals. 3′ UTR region is underlined.

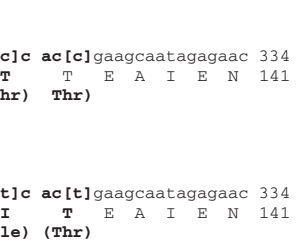

Figure 2 Part of nucleotide (from nt-299 to nt-334) and amino acid (from 130 to 141 of the mature peptide) sequences of Egyptian buffalo PCR amplified segment showing allele A $135^{Thr(acc)}/136^{Thr(acc)}$ and allele B $135^{Ile(atc)}/136^{Thr(act)}$.

(Fig. 1). Allele A has 135^{Thr}A*C*C/136^{Thr}AC*C*, whereas allele B has 135^{Ile}A*T*C/136^{Thr}AC*T* (Fig. 2).

Two haplotypes occurred in the Egyptian buffalo: $135^{Thr(ACC)}/136^{Thr(ACC)}$ and $135^{Ile(ATC)}/136^{Thr(ACT)}$. The frequency of the former haplotype is 0.57. The genotypic frequencies were 0.29 (AA), 0.65 (AB), and 0.06 (BB), whereas the allele frequencies were 0.57 (A) and 0.43 (B).

4. Discussion

In this study CSN3 exon IV has been characterized in 17 unrelated lactating Egyptian buffalo using sequence analysis. The analyzed fragment (453 nucleotide) covers 79% of CSN3 exon IV (573 bp) and 94% of its coding region (483 bp). This amplified fragment has been previously analyzed in cattle [3,4] and buffalo [31] since it covers a large percentage of the coding region. Although the number of buffalo investigated is relatively small, it verified the presence of two alleles in the Egyptian buffalo CSN3 which were previously thought to be BB monomorphic [28,21,8]. The present results showed that 2 SNPs occurred in Egyptian buffaloes at codons 135 and 136 of the mature peptide; $135^{Thr(ACC)/Ile(ATC)}$ and $136^{Thr(ACC/ACT)}$, the latter is a silent mutation. In Egyptian buffalo the nucleotide variations at both codon 135 and 136 were always the same leading to two haplotypes 135^{Thr}ACC–136^{Thr}ACC and 135^{Ile}ATC–136^{Thr}ACT. This has also been reported in the Italian buffalo by [22]. They reported a frequency of 0.579 for $135^{Thr(ACC)}/136^{Thr(ACC)}$ haplotype which is very close to what we found in the Egyptian buffalo (0.571).

The $135^{Thr/Ile}$ variation reported in the Egyptian buffalo and in the Italian buffalo [22,6] was also reported in Indian buffalo [25], and Murrah Bulgarian buffalo [5]. In Egyptian buffalo genotype AB had the highest frequency whereas in Murrah Bulgarian buffalo AA had the highest frequency. Bulgarian buffalo had the highest BB frequency (0.24) [5] when compared to Indian (0.12) [25] and Egyptian (0.06) buffaloes.

Several reports investigating the CSN3 exon IV in buffalo, by RFLP analysis, concluded that buffalo is BB monomorphic. These include Egyptian [28,21,8], Pakistani ([31], Indian [33,20], Brazilian [27] Iranian ([1] and Chinese buffaloes [30]. They all used restriction enzymes used for cattle RFLP analysis such as, HindIII and HinfI. TaqI, used in cattle RFLP analysis, was also used by [25] in RFLP analysis of buffalo.

In cattle exon IV, alleles A and B are different at codons 136 and 148 [32] and no variation occurred at codon 135 (ACC). Allele A has $136^{Thr(ACC)}/148^{Asp(GAT)}$ whereas allele B has $136^{Ile(ATC)}/148^{Ala(GCT)}$ (Fig. 3). Comparing partial nucleotide sequences (from codon 130 to149) of alleles A and B in cattle and buffalo (Fig. 3) we found that HindIII and HinfI and TaqI are not suitable for buffalo. HindIII restriction site "A^AGCTT" is present in cattle allele B (148^{Ala}) and in both buffalo alleles A and B whereas HinfI restriction site "G^ANT", contrary to HindIII, is present in cattle allele A(148^{Asp})and is missing in cattle allele B as well as in both buffalo alleles A and B. Since buffalo samples (both alleles A and B) followed cattle BB pattern, they were mistakenly assumed as BB monomorphic where in fact they would have been AA, BB or AB. TaqI restriction site T^CGA is present at codon 136 in cattle allele B (136^{Ile}) but was absent in buffalo alleles A and B (Fig. 3). Based on the above we can conclude that HindIII, HinfI and Taq1 are not suitable for buffalo RFLP analysis.

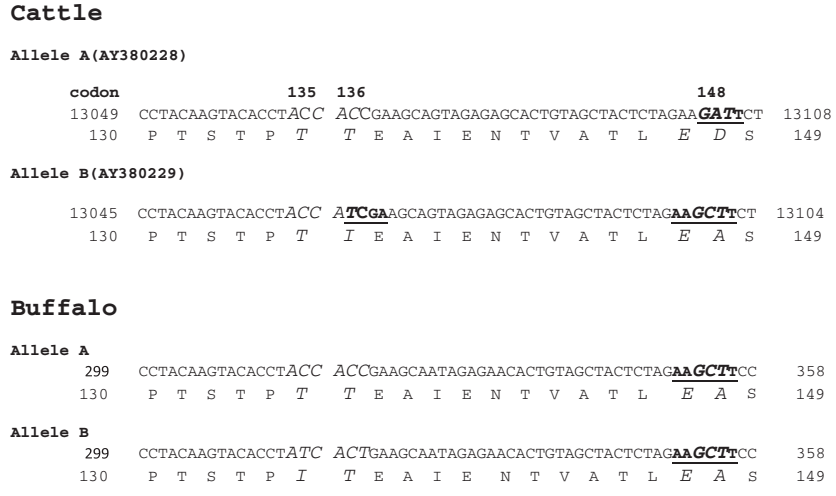

Figure 3 Partial nucleotide and amino acid sequences of cattle and buffalo alleles A and B, showing polymorphic sites (large font and *Italics*) in cattle (codons 136 and 148 of the mature protein) and in buffalo (codon135 and codon 136{silent mutation}). Restriction sites of TaqI 'T^CGA', HindIII 'A^AGCTT' and HinfI 'G^ANT' are in bold and underlined.

Figure 4 Partial nucleotide buffalo CSN3-sequence (from nt-301 to nt-360) showing AcuI and Eco57M1 restriction sites (in bold and underlined) in allele B.

As mentioned earlier in buffaloes (Egyptian buffalo and Italian buffalo [22]) two haplotypes 135^{Thr}ACC–136^{Thr}ACC and 135^{Ile}ATC–136^{Thr}ACT are present. We are here suggesting the use of restriction enzyme AcuI or Eco57M1 in buffalo RFLP analysis. The restriction sites are CTGAAGN16^ and CTGRAGN16^, respectively and are present only in buffalo allele B (Fig. 4). These enzymes are expected to digest the CSN3-exon IV 453 bp PCR amplified fragment and generate two fragments 339 bp and 114 bp in buffalo allele B. Buffalo CSN3 RFLP-analysis using the above restriction enzymes will be experimentally verified.

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